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PROVISIONAL SPECIFICATION

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Invention Title: **Porcine nuclear transfer.**

The invention is described in the following statement:

PORCINE NUCLEAR TRANSFER

This invention relates to porcine nuclear transfer processes for the production of nuclear transferred porcine embryonic cells, processes for the clonal generation of pigs, production of transgenic and genetically modified pigs, and pigs so produced.

The reconstruction of animal embryos by the transfer of a nucleus from a donor cell to either an enucleated oocyte or one cell zygote allows in theory the cloning of animals, that is, the production of genetically identical individuals. Practice is quite different. Whilst claims have been made that certain procedures have application across a wide range of animals, experience has shown that techniques which may be effective in the cloning of animals of one species either do not work in other species, give rise to embryos with a very low efficiency such that cloning would be impractical, or give rise to embryos which fail to develop on introduction to a pregnancy competent uterine environment of a recipient animal. For example, see Prather *et al*, (1989), *Biology of Reproduction* 41, 414-448.

WO 97/07668 and WO 97/07669 describe a nuclear transfer method involving donor cells resulting from serum starvation. Such donor cells, particularly embryonic cells, are substantially non-viable, having reduced biological competency as a result of serum starvation, such that they fail to develop in a pregnancy competent uterine environment in many animals, and as a consequence are generally ineffective for cloned embryo production, and development, such as in pigs.

The present invention provides processes for the high efficiency production of nuclear transferred porcine embryonic cells capable of high efficiency development in the pregnancy competent porcine uterine environment to give clonal infant animals.

In accordance with one aspect of the present invention there is provided a process for the production of nuclear transferred porcine embryonic cells which includes providing a porcine oocyte at the Metaphase II stage of development from which the nucleus is removed, transferring a porcine karyoplast at the G1 stage into the oocyte to give a nuclear

transferred porcine embryonic cell, and optionally culturing the cell *in vitro* to allow one or more cell divisions to give a plurality of nuclear transferred embryonic cells.

The nuclear transferred porcine embryonic cell may be incubated to form a 2 to 32 cell stage or mass, such as a 2 to 16 cell mass (that is, a plurality of cells), whereafter the cell mass may be synchronized at the G1 stage. A nuclear transferred karyoplast may be isolated from the cell mass, and transferred into a second enucleated oocyte at the Metaphase II stage of development to give a second nuclear transferred cell, which may be cultured *in vitro*, to allow one or more cell divisions to give a plurality of nuclear transferred porcine embryonic cells.

Karyoplasts may be synchronized at the G1 stage by use of DNA synthesis inhibitor which arrests the karyoplast at the G1 phase and/or use of a microtubule inhibitor which following removal of the microtubule inhibitor results in synchronization of said karyoplast at the G1 phase, and/or use of means which do not involve serum starvation of cells.

In another aspect this invention relates to a process for the clonal generation or propagation of pigs which process includes providing a porcine oocyte at the Metaphase II stage of development from which the nucleus is removed, transferring a porcine donor karyoplast at the G1 stage into the oocyte to give an nuclear transferred cell, culturing the nuclear transferred cell *in vitro* to allow one or more cell divisions to give a plurality of nuclear transferred porcine embryonic cells, and thereafter transferring a plurality of porcine embryonic cells so produced into a pregnancy competent uterus of a female pig which at conclusion of the pregnancy term gives rise to a plurality of genetically identical off-spring.

A further aspect of this invention provides porcine embryonic cells and pigs when prepared according to the above process.

In one aspect of this invention there is provided a process for the production of nuclear transferred porcine embryonic cells. A porcine oocyte from which the nucleus is removed is fused with the nucleus of a porcine donor karyoplast. A karyoplast is a donor nucleus,

or the nucleus of a donor cell surrounded by an envelope of cytoplasm. Porcine oocytes at the Metaphase II stage of development may be readily collected from the oviducts of ovulating pigs. Ovulation may be induced by administering gonadotrophins of various species origin to the pigs. In the practice of the present invention, oocytes can be collected on appearance of the first polar body or as soon as possible after ovulation. Alternatively immature oocytes collected from the ovaries of living or slaughtered pigs may be matured *in vitro* to the Metaphase II stage which is readily observable by microscopic evaluation.

The nucleus is removed from the porcine oocyte at the Metaphase II stage by standard techniques, such as aspiration of the first polar body and neighbouring cytoplasm containing the metaphase chromosomes (see for example Smith & Wilmut (1989) *Biol. Reprod.* 40, 1027-1035), ultraviolet radiation (see for example Tsunoda et al (1988) *J. Reprod. Fertil.* 82, 173) or another enucleating influence.

The porcine karyoplast is transferred into the porcine oocyte at the Metaphase II state as mentioned above. The karyoplast which is at the G1 stage as will be described hereinafter, is transferred into the enucleated porcine oocyte by standard techniques in the field, such as cell fusion of the enucleated porcine oocyte and the karyoplasts (that is, as mentioned above, a cell or nucleus of a cell surrounded by an envelope of cytoplasm) or by direct injection of the karyoplast into the enucleated porcine oocyte. Established methods for inducing cell fusion include exposure of cells to fusion-promoting chemicals, such as polyethylene glycol (see, for example, Kanka *et al*, (1991), *Mol. Reprod. Dev.*, 29, 110-116), the use of inactivated virus, such as sendi virus (see, for example, Graham *et al*, (1969), *Wistar Inst. Symp. Monogr.*, 9, 19), and the use of electrical stimulation (see, for example, Willasden, (1986), *Nature*, 320, (6), 63-36 and Prather *et al*, (1987), *Biol. Reprod.*, 37, 859-866). Use of electrical stimulation or cell fusion is preferred but by no means essential to this invention.

Direct micro injection of the karyoplast into an enucleated porcine oocyte may be carried out by conventional method, such as disclosed by Ritchie & Campbell, *J. Reproduction and Fertility* Abstract Series No. 15, page 60. By way of example, fusion of an enucleated

oocyte with a donor cell may be accomplished by electro-pulsing in 0.3 m mannitol or 2.7 m sucrose solution. As another example, a karyoplast may be introduced by injection into an enucleated porcine oocyte in a calcium free medium.

Enucleation of the porcine oocyte and transfer of the porcine donor karyoplast may be carried out as soon as the oocyte reaches the Metaphase II stage. This would generally coincide with the post-onset of maturation *in vitro*, after collection of ovaries from slaughtered ovulating pigs, or following hormone treatment *in vivo*.

The donor karyoplast, whether transferred directly into the cell, or transferred via fusion of the donor cell with the enucleated porcine oocyte is synchronized at the G1 stage. In this regard, the cell cycle has four distinct phases, G1, S, G2 and M, as is well known in the art. The beginning event in the cell cycle is called start which takes place at the beginning of the G1 phase. Once a cell has passed through start, it passes through the remainder of the G1 phase, which is the pre-DNA synthesis stage. The second stage, the S phase, is the stage where DNA synthesis takes place. The G2 phase follows, which is the period between DNA synthesis and mitosis. Mitosis occurs during metaphase which is referred to as the M phase.

Preferably, karyoplasts may be synchronized at the G1 stage using a DNA synthesis inhibitor and/or use of a microtubule inhibitor which, on following removal of the inhibitor(s), results in synchronization of the karyoplast at the G1 stage, or by means other than DNA inhibition, excluding serum starvation, for example cdk kinase inhibitors such as Butyrolactone I (Motlik et al (1998) *Theriogeneology* 49: 461-469). Examples of DNA synthesis inhibitors include: aphidicolin, hydroxyurea, cytosine arabinoside, 5-fluorouracil, n-ethylmalemide and etoposide. Any microtubule inhibitor may be used in this invention including nocodazole, colchecine or colcemid. Alternatively, a microtubule stabilizer such as, for example, taxol may be used. Karyoplasts may, for example, be synchronized at G1 by the use of a microtubule inhibitor such as nocodazole (to give a population of nuclei at the metaphase) followed by treatment with a DNA synthesis inhibitor such as aphidicolin in which the nuclei progress to an arrest at the G1 stage. Alternatively only one of the

aforementioned inhibitors may be utilised, or another means as discussed above which does not involve DNA synthesis inhibition.

Donor karyoplasts (in this case cells) may be incubated in a standard culture medium with a DNA synthesis inhibitor and/or microtubule inhibitor for a time sufficient to synchronize the cells at the G1 stage. This can be readily observed by microscopic observations. DNA synthesis inhibitors and/or microtubule inhibitors may be used, for example, in an amount of from about 0.01 μg/ml to about 50 μg/ml, such as about 1-5 μg/ml culture medium. Microtubule inhibitors fix the cells at the M phase. After removal of microtubule inhibitor from the cell media, which can conveniently be done by washing the cells, cells pass to the G1 phase after about 30 minutes to 6 hours in a uniform manner such that a plurality of cells in the G1 phase can be conveniently prepared. A DNA synthesis inhibitor synchronises cells at the G1 phase. Removal of a DNA synthesis inhibitor from cell media allows the cell cycle to proceed.

Donor cells may be any porcine somatic cell, for example a foetal embryonic fibroblast cell, mammary cell, smooth muscle cell etc. Any somatic cell may be utilised. The donor cell may be a porcine embryonic cell, such as a totipotent blastomere, for example a 16-32 cell mass (morula), or a cell derived from a porcine blastocyst, such as a totipotent cell from the inner cell mass of the blastocyst. The donor cell may be subject to conventional recombinant DNA manipulation where the DNA within the cell has been subject to recombinant DNA technology. For example, genes may be deleted, duplicate, activated or modified by gene additions, gene targeting, gene knock-outs, transgenesis with exogenous constructs which may or may not contain selectable markers may be accomplished by techniques such as microinjection, electroporation, viral-mediated transfection, lipofectin, calcium-phosphate precipitation (Lovell-Badge, *"Introduction of DNA into embryonic stem cells"* in: *Teratocarcinomers and Embryonic Stem Cells: A Practical Approach*, IRL Press, Oxford, E.J. Robertson, ed. pp 153-182, 1987; *Molecular Cloning: A Laboratory Manual*, Volume 2 & 3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Sambrook, Fritsch and Maniatis Ed. pp 15.3-15.50, 16.3-16.68, 1989).

The resulting nuclear transferred cell following transfer of the nucleus of the porcine donor karyoplast into an enucleated porcine oocyte may be incubated in culture medium to allow one or more cell divisions to give a plurality of porcine embryonic cells. Porcine embryonic cells as referred to herein have the capacity, on implantation into a pregnancy competent porcine uterus, to develop into a porcine foetus. Porcine embryonic cells may contain, for example, 1, 2, 4, 8, 16 or 32 cells, or more. Cell division is a relatively rapid event and can be monitored by microscopic analysis. The porcine embryonic cells may be used directly for the production of cloned pigs, or alternatively may be conveniently stored, such as by being frozen in liquid nitrogen for subsequent use.

The nuclear transferred cell may be incubated to form a 2 to 32 cell mass, such as a 2 to 16 cell mass, whereafter the cell mass is synchronized at the G1 stage as mentioned above. An nuclear transferred karyoplast may be isolated from the cell mass, and transferred into a second enucleated oocyte at the Metaphase II stage of development to give a second nuclear transferred cell, which may be cultured *in vivo* to allow one or more cell divisions to give porcine embryonic cells.

A single nuclear transferred porcine embryonic cell or plurality of cells produced according to this invention may be treated with an agent, such as cytochalasin B, so as to prevent cell division, but not nuclear division, whereafter multiple karyoplasts may be removed therefrom and used for subsequent nuclear transfer according to methods described herein (which may be regarded as serial nuclear transfer). Porcine embryonic cells as referred to herein include those treated with an agent such as cytochalasin B, or other agents.

In accordance with another aspect of this invention a nuclear transferred porcine embryonic cell or plurality of cells is treated with an agent which prevents cell division but not nuclear division, such that a karyoplast isolated therefrom is derived from a cell having multiple nuclei.

In another aspect of this invention there is provided a process for the clonal generation of pigs which process comprises providing a porcine oocyte at the Metaphase II stage of

development from which the nucleus is removed, transferring a porcine donor karyoplast at the G1 stage into the oocyte to give an NT cell, culturing the NT cell *in vivo* to allow successive cell division to give nuclear transferred porcine embryonic cells, and thereafter transferring a plurality of porcine embryonic cells so produced into a pregnancy competent uterus of a female pig which at conclusion of the pregnancy term gives rise to a plurality of genetically identical off-spring.

The clonal generation of pigs generally involves introducing into a pregnancy competent uterine environment of a female pig a plurality of embryonic cells as herein described. For example, from 5 to 50 embryonic cells may be introduced into the uterine environment according to standard procedures as used in the animal husbandry field or embryo development in gestational animals. The blastocysts may be inserted into the uterus using an appropriate device, such as a catheter or alternatively may be introduced into a fallopian tube for passage into the uterus. The recipient female animal may be primed with the embryonic cells at or about the time of ovulation which may occur naturally, or as a result of induction according to established procedures such as by administration of appropriate hormonal regimes known in the art.

According to a further aspect there are provided genetically identical pigs when prepared according to the above process.

In another aspect this invention relates to progeny of pigs produced according to this invention (which may be referred to as nuclear transfer pigs (or NT pigs)). Progeny result from crossing an NT pig with another pig to give offspring piglets, that is progeny. The other pig may be an NT pig or any other pig (for example selected for a particular trait). A progeny animal contains a part of the genetic complement of the original porcine donor karyoplast, which can be conveniently detected, for example, by DNA markers.

The present invention will now be described with reference to the following non-limiting examples.

Example 1

Collection of Oocytes from sows

Pregnant crossbred Large White X Landrace sows were aborted by intramuscular (IM) injection of 1 mg prostaglandin F2α analog (Cloprostenol; Estrumate, Pitman-Moore, NSW, Australia) between twenty five and forty days after mating followed by a second injection of 0.5 mg Cloprostenol twenty four hours later. One thousand international units of eCG (Pregnecol, Heriot AgVet, Vic, Australia) was administered (IM) at the same time as the second injection of Cloprostenol. Ovulation was induced by an IM injection of 500 iu hCG (Chorulon, Intervet, NSW, Australia) administered approximately seventy two hours after hCG. Oocytes were collected by surgically flushing oviducts forty eight to fifty two hours after hCG injection.

Culture of ova

In vitro culture of oocytes, embryos and nuclear transfer embryos was conducted in 50 μl droplets of Whitten's medium (Whitten WK, 1971, in G Raspe, ed *Advances in the Biosciences*, Pergamon Press: Oxford, pp 129-141) supplemented with 15 mg/ml bovine serum albumin (BSA) under paraffin oil in a plastic petrie dish under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 in humidified air at 38.6°C.

Example 2

Enucleation of Oocytes

Oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm (approximately 20% of cytoplasm) using a bevelled pipette (40 μm in diameter) in PB1 + 10% Fetal Calf Serum containing 7.5 μg/ml Cytochalasin B + 5μg/ml Hoechst 33342 (Sigma). Enucleation was confirmed by fluorescent staining of the aspirated portion of cytoplasm. Enucleated oocytes were cultured in Whitten's medium (WM) in a 5% CO_2 incubator until reaggregation of karyoplasts.

Reaggregation, fusion and activation of NT cells

An individual karyoplast was inserted into the perivitelline space of each enucleated oocyte. The karyoplast-oocyte complexes were cultured in WM medium until activation and fusion. Fusion and activation of the karyoplast-oocyte complexes was induced using a BTX Electro Cell Manipulator ECM 2001. The complexes were first washed in fusion medium containing 0.3M Mannitol/100μM $CaCl_2$ μM $MgSo_4$ /0.01% polyvinylalcohol and then placed between two wire electrodes (1 mm apart) of the fusion chamber (450-10WG, BTX, CA) with 0.1 ml of fusion medium. Activation and membrane fusion were induced by applying two DC pulses (150 V/mm, 60 μs) at five second intervals with a pre-, and postpulse alternating current (AC) field of 4V, 1 MHz for five seconds each).

Results obtained are shown in the following tables:

Table 1 **Metaphase arrest induced in porcine blastomere nuclei following treatment with nocodazole (NZ) dose x duration**

Duration of exposure	NZ concentration	Blastomeres at M
4 h	1 μg/ml	14/58 (24%)
7 h	1 μg/ml	54/133 (41%)
15 h	1 μg/ml	257/267 (**96%**)
15 h	0.5 μg/ml	101/133 (76%)
Control	-	15/291 (**5%**)

Table 2 ***In vitro*** **development of porcine morulae following NZ treatment**

Repeats	Duration	Dose	Development to blastocyst
4	15 h	1 μg/ml	17/30 (57%)
3	15 h	0.5 μg/ml	14/20 (70%)
3	15 h	Control	15/20 (75%)

Table 3 **Nuclear transfer results using karyoplasts at three different stages of the cell-cycle**

Karyoplast stage	Cytoplast stage	No. reps.	n	2-cell (%)	4-cell (%)	Morula (%)	Blastocysts (%)
S-phase	S	7x	159	85 **(54)**	35 **(22)**	16 **(10)**	6 **(4)**
Metaphase	M II	3x	40	29 **(73)**	10 **(25)**	2 **(5)**	0 **(0)**
G1	M II	4x	42	30 **(71)**	20 **(48)**	12 **(29)**	6 **(14)**

Example 3

Embryo Transfer of Nuclear Transfer Embryo

Pregnant crossbred Large White X Landrace sows are aborted by intramuscular (IM) injection of 1 mg prostaglandin F2α analog (Cloprostenol; Estrumate, Pitman-Moore, NSW, Australia) between twenty five and forty days after mating followed by a second injection of 0.5 mg Cloprostenol twenty four hours later. Five hundred international units of eCG (Pregnecol, Heriot AgVet, Vic, Australia) is administered (IM) at the same time as the second injection of Cloprostenol. Ovulation is induced by an IM injection of 500 iu hCG (Chorulon, Intervet, NSW, Australia) administered approximately seventy two hours after eCG. Twenty-five to thirty, 4-cell embryos surgically transferred to the oviduct of a sow seventy two hours after the hCG injection result in a litter of 5 to 8 piglets following a successful pregnancy.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" or the term "includes" or variations thereof, will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers. In this regard, in construing the claim scope, an embodiment where one or more features is added to any of claims is to be regarded as within the scope of the invention given that the essential features of the invention as claimed are included in such an embodiment.

THE CLAIMS:

1. A process for the production of nuclear transferred porcine embryonic cells which includes providing a porcine oocyte at the Metaphase II stage of development from which the nucleus is removed, transferring a porcine karyoplast at the G1 stage into the oocyte to give a nuclear transferred porcine embryonic cell and optionally culturing the nuclear transferred cell *in vitro* to allow one or more cell divisions to give a plurality of nuclear transferred porcine embryonic cells.

2. A process according to claim 1 wherein the nuclear transferred porcine embryonic cell or plurality of cells, such as a 2 to 32 cell mass, is synchronized at the G1 stage, isolating a nuclear transferred karyoplast therefrom, and transferring said karyoplast into a second enucleated oocyte at the Metaphase II stage of development to give a second nuclear transferred cell, which may be cultured *in vitro* to allow one or more cell divisions to give a plurality of nuclear transferred porcine embryonic cells.

3. A method according to claim 2 wherein the nuclear transferred porcine embryonic cell or plurality of cells is treated with an agent which prevents cell division but not nuclear division, such that a karyoplast isolated therefrom is derived from a cell possessing multiple nuclei.

4. A method for the production of porcine embryonic cells wherein the method of claim 3 is repeated a plurality of times.

5. A process for the clonal generation or propagation of pigs which process includes providing a porcine oocyte at the Metaphase II stage of development from which the nucleus is removed, transferring a porcine donor karyoplast at the G1 stage into the oocyte to give a nuclear transferred porcine embryonic cell, and thereafter culturing the nuclear transferred cell *in vitro* to allow one or more cell divisions to give a plurality of nuclear transferred porcine embryonic cells, and thereafter transferring a plurality of porcine embryonic cells so produced into a pregnancy competent uterus of

a female pig which at conclusion of the pregnancy term gives rise to a plurality of genetically identical off-spring.

6. A process according to claim 1 wherein a karyoplast is synchronized at the G1 stage by use of DNA synthesis inhibitor and/or a microtubule inhibitor and/or use of means which do not involve serum starvation of cells.

7. A process according to any of claims 1 to 5 in which the karyoplast is genetically altered or modified.

8. A process according to claim 6 where microtubule inhibition is achieved by the application of nocodazole.

9. A process according to claim 1 wherein karyoplast synchronization at G1 is achieved by the application of aphidicolin.

10. Porcine embryonic cells or cloned pigs when produced according to a process comprising or including a process as defined in any preceding claim.

11. Progeny of a pig according to claim 10.

DATED this 16th day of March, 1998.

BRESAGEN LIMITED
By Its Patent Attorneys
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